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Journal of Neurological Sciences 151 (1997) 41–48

JOURNAL OF THE NEUROLOGICAL SCIENCES

Identification and characterization of an anti-glial fibrillary acidic protein antibody with a unique specificity in a demented patient with an autoimmune disorder

Kazuyuki Ishida[a], Kiyotoshi Kaneko[a], Tetsuo Kubota[b], Yoshinori Itoh[c], Tadashi Miyatake[a], Masaaki Matsushita[d], Masahito Yamada[a,*]

[a]*Department of Neurology, School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan*
[b]*School of Allied Health Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan*
[c]*Department of Internal Medicine, Yokuhukai Geriatric Hospital, Tokyo, Japan*
[d]*Department of Psychiatry, School of Medicine, University of Tokyo, Tokyo, Japan*

Received 21 November 1996; revised 19 March 1997; accepted 24 March 1997

Abstract

We detected an antibody to a 48 kd antigen of the central nervous system in the serum from a demented patient with an autoimmune disorder. To identify and characterize the antigen, we screened a human cerebral cDNA library and performed immunoblot analysis following two-dimensional gel electrophoresis (2-D blotting). The sequences of the isolated cDNA fragments were homologous to human glial fibrillary acidic protein (GFAP). Two-D blotting using patient serum revealed that the antibody reacted with a restricted subset of GFAP molecules which exhibited relatively high isoelectric points. Furthermore, to elucidate the importance of the anti-GFAP antibody in dementia, we screened for the presence of an anti-GFAP antibody in the sera of 46 demented patients: 26 with Alzheimer's disease and 20 with vascular dementia (VD). We found an anti-GFAP antibody in the serum of only one patient with VD. Two-D blotting revealed that the anti-GFAP antibody in the serum from the VD patient reacted with a more acidic subset of GFAP molecules compared with the anti-GFAP antibody from our patient. In conjunction with the fact that the GFAP molecule with high isoelectric point was insoluble and less degraded, these results suggested that the anti-GFAP antibody in the serum of our patient was not generated due to a secondary response to soluble and degraded GFAP which leaked through the damaged blood–brain barrier as found in the VD patient, but was generated actively on the basis of dysregulation of the immune system. Possible effects of the autoantibody on astrocytic function and the pathogenesis in dementia are discussed. © 1997 Elsevier Science B.V.

Keywords: Autoimmune disorder; Alzheimer's disease; Vascular dementia; Anti-GFAP antibody; cDNA cloning; Two-dimensional gel electrophoresis; Isoelectric point

1. Introduction

Dementia may be caused by immune-mediated mechanisms either solely or in association with other autoimmune disorders. For example, Rasmussen's encephalitis is reported to have a causative relationship to anti-glutamate receptor antibody (Rogers et al., 1994). Paraneoplastic limbic encephalitis, which is characterized by severe impairment of recent memory, is associated with anti-Hu antibody (Anderson et al., 1987; Posner, 1991). Caselli et al. (1991, 1993) reported on two demented patients with primary Sjögren's syndrome (SS). The patients were first diagnosed as having Alzheimer's disease (AD), but treatment with prednisone was successful in alleviating their symptoms, indicating the similarity of the symptoms of AD to those of chronic meningoencephalitis which occurs in SS. Understanding of the mechanisms underlying immune-mediated or autoimmune dementia is important

*Corresponding author. Tel. and fax: +81 3 58035234.

PII S0022-510X(97)00108-1

because it may be treatable and sometimes reversible. Dubovik et al. (1993) reported on experimental autoimmune dementia (EAD) generated in rats by immunization with the high molecular weight neurofilament protein, one of the intermediate filaments, to establish an animal model of dementia. EAD is not only an animal model of autoimmune dementia but important evidence that such an intracellular protein can be recognized and attacked by the immune system. Furthermore, in patients with AD, immune mechanisms seem to contribute to the progress of dementia (Serot et al., 1992; Heinonen et al., 1993). In fact, non-steroidal anti-inflammatory drugs are reported to be effective in some patients diagnosed as having AD (Rich et al., 1995).

We found a circulating antibody to a 48 kd antigen of the central nervous system (CNS) in a patient with an autoimmune disorder and dementia that clinically resembled AD (Kaneko et al., 1993). We hypothesized that an immune response to the antigen might play a role in the development of dementia. We also speculated that the antigen might be involved in the pathogenesis of AD. Herein, we describe cloning of the cDNA encoding the 48 kd antigen and characterization of the immunochemical properties of the antigen. We demonstrated that the antigen belongs to a unique subset of glial fibrillary acidic protein (GFAP) molecules and speculate that it might be related to the pathogenesis of the dementia in our patient.

2. Materials and methods

2.1. Patient report

The clinical course of dementia in the patient has been reported elsewhere (Kaneko et al., 1993). Briefly, a 54 year old man suffered from progressive impairment of memory and mental activity over a two year period. Neurological examination revealed memory disturbance and construction apraxia. His IQ was 45 on the Suzuki–Binet scale. Laboratory examinations revealed the presence of anti-nuclear antibody (ANA) and anti-ribonucleoprotein (RNP) antibody in his serum, but the patient's symptoms did not meet the criteria for diagnosis of classical autoimmune disease. Examination of his cerebrospinal fluid (CSF) revealed lymphocytic pleocytosis (68/mm^3) and elevated protein concentration (98 mg/dl) with high immunoglobulin G (IgG) index (36%). A magnetic resonance imaging scan of his head revealed mild atrophy of the frontal and temporal lobes. Oral prednisolone therapy (60 mg/day) did not alleviate the neurological impairments in spite of an improvement in the IgG content in the CSF and in the serum ANA titer.

The patient was first diagnosed as having AD, but the initial diagnosis was amended to autoimmune dementia because of the presence of an autoantibody to a 48 kd antigen of the CNS in his serum and the CSF and the abnormal findings in the CSF.

2.2. cDNA cloning strategy

In addition to the antibody to the 48 kd CNS antigen, the serum from the patient contained antibodies against constituent proteins of *Escherichia coli* in large quantities and autoantibodies such as ANA and anti-RNP antibody. The anti-*E. coli* antibodies could not be removed from the serum by pseudoscreening or column chromatography due to their high concentration (Sambrook et al., 1989). Therefore, we could not perform immunological screening to identify the target antigen using the patient's serum as a probe (Sambrook et al., 1989). Thus we screened a cDNA library using an alternative strategy as shown in Fig. 1. Using this method, we carried out immunoscreening without the influence of autoantibodies and anti-*E. coli* antibodies.

2.2.1. Tissue samples

Samples of bovine nervous system tissue (cerebrum, cerebellum and spinal cord) were obtained from a local slaughterhouse. Samples of human cerebrum were obtained at autopsies of neurologically normal patients. The autopsies were performed within 10 h after patients' deaths. Samples were immediately frozen and stored at −80°C until use.

2.2.2. Western blot analysis

Tissue samples were homogenized with lysis buffer [20 m*M* Tris–HCl pH 7.4, 0.1% sodium dodecyl sulfate

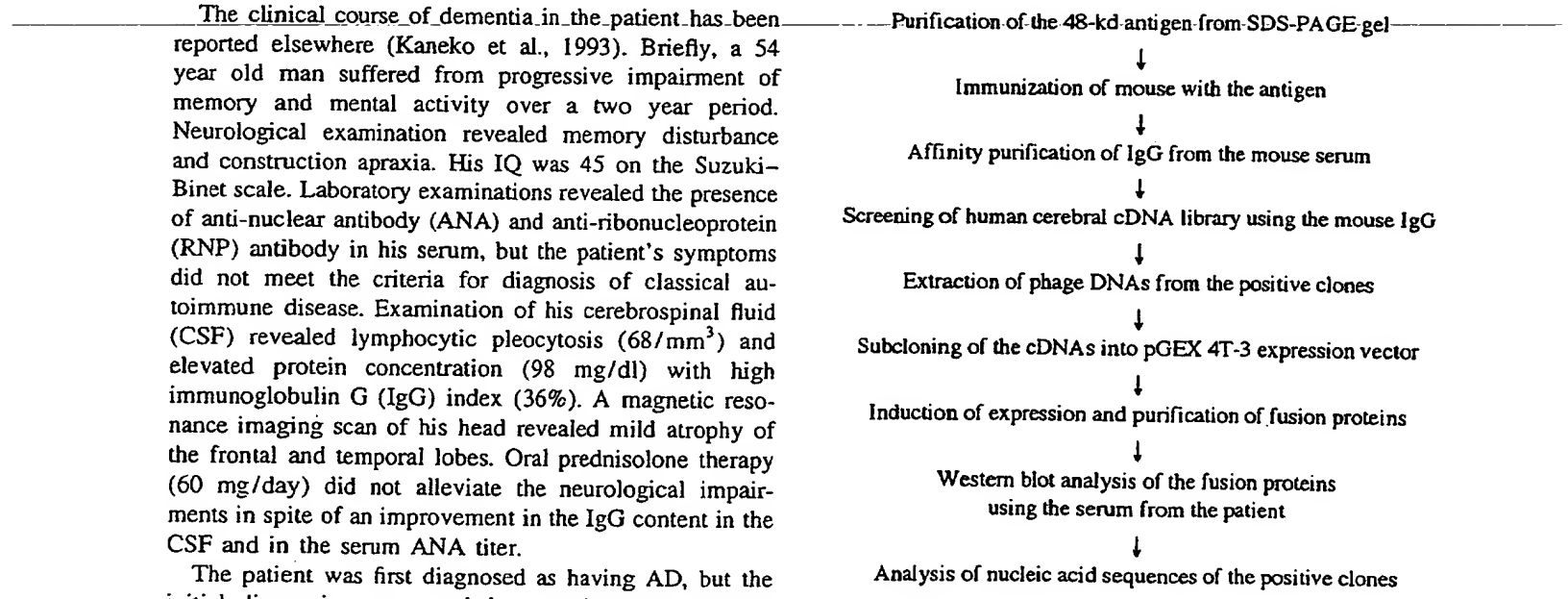

Fig. 1. Strategy for cloning of the cDNA encoding the 48 kd CNS antigen.

(SDS), 1% Triton X-100, 1% sodium deoxycholate] and the protein concentration of the homogenate was determined by the Bradford method (Bradford, 1976). These samples were mixed with modified Laemmli's sample buffer (2% SDS, 20% sucrose, 20 m*M* Tris, 2 m*M* EDTA, 80 m*M* dithiothreitol (DTT) and then subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Separated proteins were electrophoretically transferred to nitrocellulose membranes (Towbin et al., 1979). The membranes were incubated with the diluted human sera or mouse antibody for 1 h, and after three washes, reacted with peroxidase-conjugated goat antibody to human IgG (Sigma, St. Louis, MO, USA) or mouse IgG (Sigma), respectively. Immunoreactivity of the antigens on the membranes was visualized by an enhanced chemiluminescence western blotting detection system (Amersham, Bucks, UK).

2.2.3. Two-dimensional gel electrophoresis (2-D EP) and immunoblot analysis following 2-D EP (2-D blotting)

Bovine cerebral proteins were separated by 2-D EP using a Pharmacia Multiphor II unit (Pharmacia Biotech, Uppsala, Sweden) as described by Bruun et al. (1993). For the first-dimension electrophoresis, 1000 μg of cerebral sample, homogenized in 2-D EP sample buffer, was loaded at the anodic side of the 11 cm DryStrip 4-7. An isoelectric focusing step was performed at a final voltage of 3500 V for 20 h. The second-dimension electrophoresis was performed on a Pharmacia ExcelGel 12.5% gel following standard procedures. Separated proteins were electrophoretically transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Immunostaining of the membrane was performed as described for the western blot analysis.

2.2.4. Purification of antigen and immunization of mice

Human cerebral sample was subjected to SDS-PAGE and the gel was stained with Coomassie brilliant blue. The band in the gel corresponding to the band labeled by immunostaining was cut out and the protein in the gel slices was electrophoretically recovered (Hunkapiller et al., 1984). A 10 μg weight of the purified protein was dissolved in 100μl of phosphate buffered saline (PBS), mixed with the same volume of complete adjuvant and injected intraperitoneally into female BALB/C mice. At intervals of three weeks the mice were inoculated with the antigen in conjunction with incomplete adjuvant for a total of four times. After the last inoculation, we confirmed that a high titer antibody to the 48 kd antigen had been raised in the mice sera by western blotting.

2.2.5. Purification of mouse IgG

The IgG fraction of the mice sera was purified by protein G Sepharose column chromatography (Pharmacia). Anti-*E. coli* antibody in the IgG fraction was removed by loading the fraction onto a CNBr-activated Sepharose column previously coupled with constituent proteins of *E. coli*. Removal of anti-*E. coli* antibody was confirmed by western blotting using lysate of *E. coli* as an antigen. The purified IgG was stored at 4°C with 0.02% sodium azide as a preservative until use. The IgG was diluted with Tris-buffered saline (TBS) to 10μg/ml and used as a probe for immunoscreening.

2.2.6. Screening of a cDNA library

We screened a lambda zap human cerebral cDNA expression library (Stratagene, La Jolla, CA, USA) with the mouse IgG. According to the manufacturer's information, this library was constructed using mRNA extracted from cerebral cortex of a 85-year-old female. The recombinant phages (2×10^6 plaque forming units) were plated on agar plates seeded with *E. coli* strain XL1-Blue and incubated at 42°C for 3.5 h. Then the plates were overlaid with filters soaked in 10 m*M* isopropyl-β-D-thiogalactoside (IPTG) which was used for induction of expression of fusion proteins, and incubated at 37°C for an additional 3.5 h. The filters were washed in TBS, and incubated with the purified mouse IgG for 1 h at room temperature and then with diluted alkaline phosphatase-conjugated anti-mouse IgG (1:1000 dilution, Bio-Rad, Hercules, CA, USA) for 1 h. After washing in TBS, the filters were stained by nitroblue tetrazolium chloride and bromochloroindolylphosphate solution (Sambrook et al., 1989), and positive plaques of the recombinant phages were recovered from the agar plates. We repeated these procedures until 100% of the plaques gave positive signals.

2.2.7. Subcloning of the cDNA into plasmid vectors

The cDNA fragments carried in the positive clones identified from the screening of the cDNA library were subcloned into pBluescript vector using an in vivo excision protocol according to the manufacturer's instructions for the lambda zap cDNA library. The subcloned cDNAs were further subcloned into pGEX 4T-3 expression vector (Pharmacia) using the T-A overhang cloning method (Finney, 1995). We considered that the T-A cloning method would prevent the inserts from unexpected digestion by restriction endonucleases because we did not know the sequences of the inserts at this time. The cDNAs within pBluescript vectors were amplified by the polymerase chain reaction method using a pair of vector-based primers (SK and KS primer, Stratagene) and purified by low melting point agarose gel electrophoresis and subsequent recovery of the amplified fragments from the gel (Sambrook et al., 1989). An expression vector was prepared by digestion using Sma I restriction endonuclease and addition of a single T to the 3′ end of the digested vector using Taq DNA polymerase. The purified cDNAs were ligated into the prepared vector with the T overhang. After ligation, all plasmids were transformed into *E. coli* XL1-Blue.

2.2.8. Induction of expression and analysis of fusion proteins encoded by the cDNAs

The glutathione-S-transferase (GST) gene fusion system is a bacterial expression system to express foreign proteins as fusions with GST. Fusion proteins can be purified from bacterial lysate by affinity chromatography using glutathione–agarose beads (Smith and Corcoran, 1994). The transformed clones were grown in liquid media containing 100 μg/ml ampicillin up to an optical density of 0.6 and the expression of fusion proteins was induced by addition of IPTG to a concentration of 1 m*M*. After two hours of incubation at 37°C, the cells were harvested by centrifugation and resuspended in PBS containing 0.1% Triton X-100 and 0.25% sarcosine, and sonicated. The lysates were centrifuged to remove insoluble material. Glutathione Sepharose beads (Pharmacia) were added to the supernatants to absorb the fusion proteins. After four washes in PBS, the fusion proteins were eluted from the beads by addition of 10 m*M* glutathione solution.

The purified fusion proteins were analyzed by western blotting using the patient serum and the mouse antibody. When the fusion proteins reacted with both probes, we regarded the clones which expressed the fusion proteins as the clones which carried cDNA fragments encoding the 48 kd antigen.

2.2.9. DNA sequence analysis

Sequencing was performed according to the dye–dideoxy termination method using a 373A automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA). The cDNAs carried on the pBluescript vector in the positive clones were sequenced for both strands using M13 universal, reverse primers and internal oligonucleotide primers. Sequences were analyzed for homology to known cDNA sequences using GenetyxMac software (Software Development, Tokyo, Japan) to search the Genbank® database.

2.3. Absorption assay

Sequence analysis revealed that the cloned cDNAs were fragments of the GFAP cDNA (Reeves et al., 1989). Thus absorption of the patient serum with GFAP was performed to confirm that the 48 kd antigen was GFAP. Purified GFAP, purchased from Progen Biotechnik (Heidelberg, Germany), was incubated with the patient serum (1:2000 dilution) overnight at 4°C. After incubation, the reaction mixtures were then centrifuged and the supernatants were tested for decrease in immunoreactivities against the 48 kd antigen by western blotting. We also used a polyclonal anti-GFAP rabbit antibody (Dako, Copenhagen, Denmark) as a standard antibody for control studies.

2.4. Screening for the presence of a circulating anti-GFAP antibody in the sera of demented patients

To investigate the role of anti-GFAP antibody in the pathogenesis of dementia, we screened for the presence of a circulating anti-GFAP antibody in the sera of demented patients and control subjects. Serum samples were obtained from 26 patients with AD including both presenile and senile onset AD, 20 patients with VD, 12 subjects with diseased conditions (8 patients with spinocerebellar degeneration and 4 patients with meningoencephalitis with systemic lupus erythematosus or SS) and 25 normal controls. Diagnosis of AD or VD was neuropathologically established at postmortem examinations. All serum samples were tested by western blotting using the purified GFAP as an antigen. Before screening, we determined an optimal dilution of serum samples (1:4000 dilution) that gave distinct reactivity of the patient serum with GFAP but no non-specific reactivity of control sera with GFAP.

3. Results

On western blotting using bovine samples as antigens, the serum of our patient binds to a 48 kd antigen in the cerebrum, cerebellum and spinal cord, but did not react with proteins from the liver (Fig. 2). At a 1:2000 dilution of the serum, we did not detect immunoreactivities of ANA or anti-RNP antibody on the western blotting. However, we confirmed that western blot showed additional bands which might represent systemic autoantibodies when the serum was applied at a lower dilution. We extracted the 48 kd antigen from polyacrylamide gel and inoculated it into mice. On a western blotting, the serum from the immunized mice exhibited strong immunoreactivity with the 48 kd antigen (Fig. 3). There were many additional bands of lower molecular weight which were also immunostained. These were probably degra-

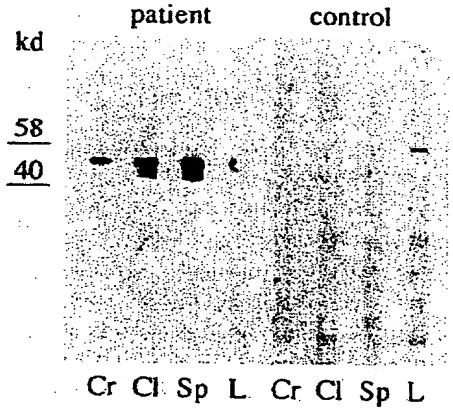

Fig. 2. Western blotting of bovine samples stained with the patient serum and control serum (1:2000 dilution). Immunoreactivities with a 48 kd antigen are observed in the lanes of the cerebrum (Cr), cerebellum (Cl) and spinal cord (Sp) but not in the lane of the liver (L). There are some faint bands of lower molecular weights which seem to be degradation products of the 48 kd antigen. The immunoreactivities are detectable at least up to a 1:10 000 dilution of the patient serum. The control serum reacted with an unknown band in the lane of L, which we did not investigate further.

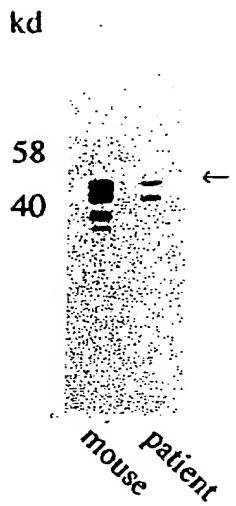

Fig. 3. Western blotting of the bovine cerebral proteins stained with the serum from the mouse immunized with the 48 kd antigen (1:10 000 dilution) and the patient serum (1:2000 dilution). Strong immunoreactivity with the 48 kd band (arrow) with additional bands of smaller molecular weights can be seen.

dation products of the 48 kd antigen. However, we could not deny the possibility that additional bands might represent contaminant proteins present in the antigen preparation used for immunization of the mice or other proteins carrying similar epitopes to GFAP.

We screened 2×10^6 recombinants of a human cerebral cDNA expression library using the purified mouse IgG as a probe and isolated twenty positive clones of lambda phages. The cDNA fragments carried in these twenty clones were subcloned into pBluescript vector and then into pGEX 4T-3 expression vector, and the expression of GST-tagged fusion proteins was induced. We screened isolated fusion proteins by western blotting and identified two independent clones (clone 1 and clone 2) encoding the fusion proteins recognized by both the mouse IgG and the patient serum. The plasmids, clone 1 and clone 2, had inserts of approximately 1.2 kilobases (kb) and 2.8 kb, respectively.

A homology search of the sequences in the Genbank database revealed that the inserts in both clones consisted of fragments of the cDNA encoding human GFAP (Fig. 4). The sequence of each insert was 100% homologous to the corresponding part of the GFAP cDNA. Both inserts started within the open reading frame (ORF) of the GFAP cDNA and contained a stop codon. Clone 2 contained a polyA stretch in the 3′ terminal region, but clone 1 stopped immediately after the stop codon of the GFAP cDNA.

To further confirm that the 48 kd band on the western blotting represented GFAP, the patient serum was absorbed with the purified GFAP prior to western blotting. The immunoreactivity of the patient serum with the 48 kd antigen was clearly reduced after the absorption as found on western blotting of the standard anti-GFAP antibody absorbed with the purified GFAP (Fig. 5).

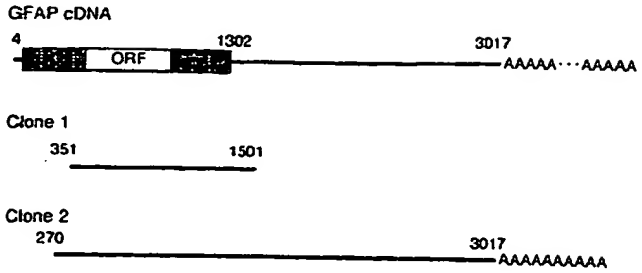

Fig. 4. Schematic illustration showing relationship between human GFAP cDNA and the cloned cDNAs. The complete sequences and the open reading frame (ORF) of the GFAP cDNA is shown at the top of this figure. Both inserts started within ORF of the GFAP cDNA (position 351 and 270, respectively) and contained a stop codon of the GFAP cDNA (position 1302). Clone 2 contained a polyA stretch in the 3′ terminal region (position 3017), but clone 1 stopped at position 1501 of the GFAP cDNA, immediately after the stop codon. Shaded box represents ORF of the GFAP cDNA. Numbers represent positions in the sequence of the GFAP cDNA.

To characterize the anti-GFAP antibody contained in the patient serum, we performed 2-D blotting and compared the immunostaining pattern using the patient serum with that using the standard anti-GFAP antibody (Fig. 6). The standard antibody reacted with multiple spots which represented GFAP molecules which had the same molecular weight but different isoelectric points (p*I*s). The p*I*s of the spots varied widely. There were many degradation products with lower molecular weights than intact GFAP. These degradation products appeared to be increasingly acidic with decreasing molecular weight as reported by Dahl et al. (1982); Bigbee et al. (1983). In contrast, immunoreactivity of the patient serum was restricted to a subset of GFAP molecules with relatively high p*I*s.

Furthermore, we screened for the presence of a circulating anti-GFAP antibody in the sera of 26 AD patients, 20 VD patients, 12 subjects with diseased conditions and 25 normal controls. Among them, only one VD patient had an anti-GFAP antibody. Interestingly, on a 2-D blotting, the serum from the VD patient reacted with a subset of GFAP molecules which was more acidic than the subset with which the serum from our patient reacted (Fig. 7).

4. Discussion

In this study, sequence analysis of the cloned cDNAs revealed that the 48 kd antigen recognized by the patient serum was GFAP. The results of the absorption assay using purified GFAP confirmed that the anti-CNS antibody in the serum of our patient reacted with GFAP. On 2-D blotting, the anti-GFAP antibody of our patient was characterized by its unique immunostaining pattern. The serum of our patient reacted with a restricted subset of GFAP molecules with relatively high p*I*s in contrast with the standard antibody or the serum from the VD patient which reacted with a more acidic subset. In addition, we performed immunostaining of bovine cerebral sections to investigate

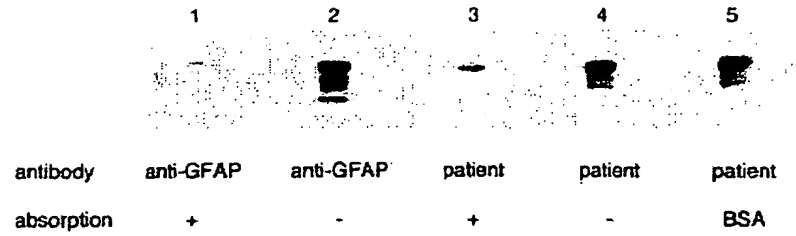

Fig. 5. Absorption of the serum with GFAP. The serum from the patient and a standard anti-GFAP antibody (Dako, Copenhagen, Denmark) were absorbed with GFAP (0.2 mg/ml) prior to western blotting. Immunoreactivities of the patient serum and standard antibody were markedly decreased by absorption with GFAP. Lanes: (1) standard anti-GFAP antibody absorbed with GFAP; (2) standard antibody without absorption; (3) the patient serum absorbed with GFAP; (4) the patient serum without absorption; (5) the patient serum absorbed with bovine serum albumin (BSA, 0.2 mg/ml) instead of GFAP.

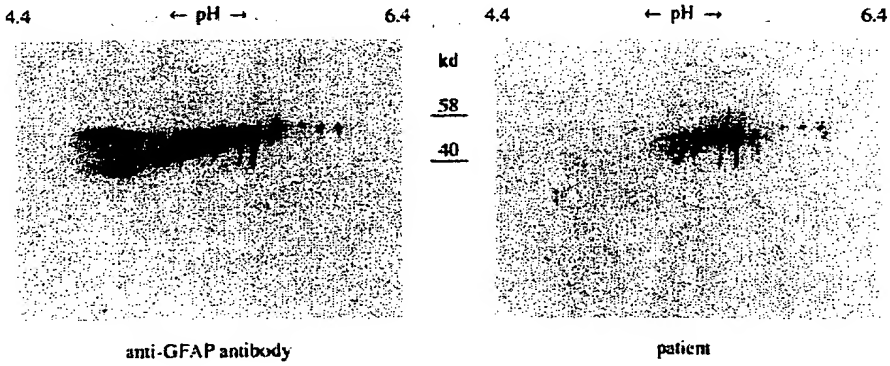

Fig. 6. Immunoblotting following two-dimensional gel electrophoresis (2-D blotting) using the patient serum and the standard anti-GFAP antibody as a probe. The standard anti-GFAP antibody binds to the 48 kd antigens of the wide p*I* range. The patient serum reacts with a subset of the 48 kd antigens of higher p*Is*.

histologically the specificity of the serum; the serum reacted strongly with the nuclei of neurons and astrocytes but not clearly with the cytoplasm of astrocytes (data not shown). This strong nuclear staining seemed to represent the presence of ANA in the serum of our patient.

It is known that GFAP molecules migrate as multiple spots with the same molecular weight but different p*Is* on 2-D EP (Bigbee and Eng, 1982). These differences in the p*Is* of GFAP molecules depend on the degree of phosphorylation (Inagaki et al., 1994). The higher the degree of phosphorylation of a GFAP molecule, the more acidic it becomes. The solubility of GFAP is also correlated to the degree of phosphorylation (Inagaki et al., 1994). The anti-GFAP antibody of our patient seems to react with GFAP molecules with a low degree of phosphorylation and low solubility. If the anti-GFAP antibody of the patient was raised in response to the leakage of soluble GFAP through the damaged blood–brain barrier (BBB) as a result of a CNS disorder, the antibody would react with soluble rather than insoluble GFAP. Indeed, the anti-GFAP antibody from the VD patient reacted with the acidic and soluble subset of GFAP molecules. This result also supported our hypothesis that the anti-GFAP antibody in our patient was not generated in response to soluble GFAP but

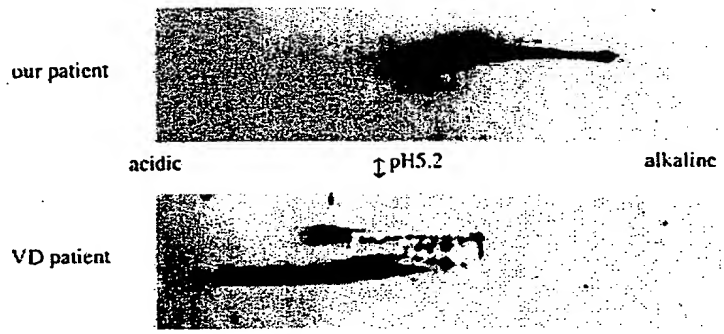

Fig. 7. Two-D blotting stained with the serum from the VD patient who had a circulating anti-GFAP autoantibody in his serum. The serum from the VD patient reacts with a subset of GFAP molecules which is more acidic than the subset with which the serum from our patient reacts.

was generated actively against insoluble GFAP. On the other hand, the result that the immunoreactivity of the anti-GFAP antibody of our patient was not detected upon immunohistochemical analysis, despite the strong immunoreactivity on the western blotting, suggests that the epitope of the antigen may be concealed in situ due to intra- or intermolecular conformation. To be recognized by the immune system, the hidden epitope must be exposed. However, the exposure process did not seem to be a mere degradation process, because, if exposure of epitope depended simply upon a degradation process, immunoreactivity of the anti-GFAP antibody must be stronger in proportion as GFAP molecules become more acidic and smaller on 2-D blotting. The autoantibody to an insoluble subset of GFAP molecules might be actively generated on the basis of dysregulation of the immune system in association with a systemic autoimmune disorder. Molecular mimicry to invading organisms may be an alternative etiology of generation of the anti-GFAP antibody. However, we do not consider that this is the case because the patient had no evidence of infection before or at the onset of dementia.

In addition to our patient, we found an anti-GFAP antibody in the serum of only one patient with VD but in the serum of none with AD in this study. There have been several reports regarding the presence of a circulating anti-GFAP antibody in the sera of demented patients. Tanaka et al. (1988) reported a high incidence (80%) of the presence of an anti-GFAP antibody in the sera of AD patients, whereas Mecocci et al. (1992), (1995) reported that the level of an anti-GFAP antibody was higher in the sera of VD patients than in the sera of AD patients, attributing the generation of anti-GFAP antibody to an alteration of the BBB in VD patients. The discrepancy between such previous reports and ours may be due to the purity of GFAP used in screening for the presence of an anti-GFAP antibody and the sensitivity of the screening. In ours and previous studies, isolated GFAP was used as an antigen for the screening. However, the isolated GFAP necessarily contained a small amount of contaminating proteins (Dahl and Bignami, 1973; Liem and Shelanski, 1978; Rueger et al., 1978) which might cause false–positive reactions in enzyme-linked immunosorbent assay (ELISA). In this study, we chose to use western blotting instead of ELISA in order to distinguish the GFAP band from other bands representing contaminating proteins. The sensitivity of western blotting is generally lower than that of ELISA. In our screening study, we deliberately adjusted the western blotting sensitivity to a lower level than usual in order to obtain a high selectivity and to prevent nonspecific reactions. The differences in the method used and the screening assay conditions may be reasons for differences in the incidence of the detection of an anti-GFAP antibody in the sera of demented patients.

GFAP is one of the brain-specific proteins in astrocytic glia and the major component of gliosed tissue (Bignami et al., 1980; Steinert and Roop, 1988), but its function has not yet been completely elucidated. Weinstein et al. (1991) demonstrated that the suppression of GFAP expression by antisense mRNA treatment resulted in the inability of formation of stable astrocytic processes. Liedtke et al. (1996) reported that GFAP-deficient mice generated by gene targeting exhibited incomplete myelination in the optic nerve and inability of septation of the white matter in the spinal cord. However, other researchers reported that lack of GFAP had no effect upon development and behavior of GFAP-deficient mice (Pekny et al., 1995; Shibuki et al., 1996). Although it is still not clear whether the lack of GFAP itself can cause dementia, astrocytes have important roles in forming the BBB and maintaining an appropriate environment for neurons (Manthorpe et al., 1986; Janzer and Raff, 1987). Yamada et al. (1991) reported a possible role of anti-astrocyte antibody in AIDS dementia complex, although the astrocytic antigen was not GFAP but a 43 kd protein. We speculate that ill effects on astrocytes by the anti-GFAP antibody may lead to neuronal dysfunction, and then to dementia.

For immunoscreening of a cDNA library, we used IgG purified from the serum of a mouse immunized with the 48 kd antigen isolated by SDS-PAGE, because the patient serum contained other autoantibodies and anti-*E. coli* antibody in large quantities. In general, if a suitable antibody is not available for immunoscreening, protein microsequencing will be used instead of cDNA screening. In our case, however, we could not use protein microsequencing. On 2-D blotting using the serum of our patient, multiple spots connected to each other by streaks were observed and it was difficult to identify the target antigen of the antibody. Our cloning method is unusual and complicated, but it will be effective if common methods are not suitable.

In conclusion, we demonstrated that an antibody to a 48 kd CNS antigen in a demented patient with an autoimmune disorder recognized a unique subset of GFAP molecules. The antibody might be generated actively on the basis of dysregulation of the immune system. The antibody might impair astrocytic function and play a role in the pathogenesis of the immune-mediated dementia. This is the first report of heterogeneity in the specificity of anti-GFAP antibodies in demented patients. Although further investigations are necessary to elucidate the pathomechanism underlying the immune-mediated dementia, our findings concerning the diversity of the anti-GFAP antibody should facilitate its characterization.

Acknowledgments

We are grateful to Prof. H. Mizusawa (Department of Neurology, School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan) for careful reading of the manuscript. This study was supported in part by Health

Science Research Grants (Mechanism of Abnormal Deposition in Dementia Brain) of the Ministry of Health and Welfare of Japan.

References

Anderson, N.E., Cunningham, J.M., Posner, J.B., 1987. Autoimmune pathogenesis of paraneoplastic neurological syndromes. Crit. Rev. Neurobiol. 3, 245–299.

Bigbee, J.W., Eng, L.F., 1982. Analysis and comparison of in vitro synthesized glial fibrillary acidic protein with rat CNS intermediate filament proteins. J. Neurochem. 38, 130–134.

Bigbee, J.W., Bigner, D.D., Pegram, C., Eng, L.F., 1983. Study of glial fibrillary acidic protein in a human glioma cell line grown in culture and as a solid tumor. J. Neurochem. 40, 460–467.

Bignami, A., Dahl, D., Rueger, D.C., 1980. Glial fibrillary acidic protein (GFA) in normal neural cells and in pathological conditions. In: Fedoroff, S., Hertz, L. (Eds.), Advances in Cellular Neurobiology, vol. 1. Academic Press, New York, pp. 285–310.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Bruun, C.F., Sletten, K., Husby, G., Marhaug, G., 1993. Characterization and quantification of mink serum amyloid A protein using two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 14, 1372–1374.

Caselli, R.J., Scheithauer, B.W., Bowles, C.A., Trenerry, M.R., Meyer, F.B., Smigielski, J.S., Rodriguez, M., 1991. The treatable dementia of Sjögren's syndrome. Ann. Neurol. 30, 98–101.

Caselli, R.J., Scheithauer, B.W., O'Duffy, J.D., Peterson, G.C., Westmoreland, B.F., Davenport, P.A., 1993. Chronic inflammatory meningoencephalitis should not be mistaken for Alzheimer's disease. Mayo Clin. Proc. 68, 846–853.

Dahl, D., Bignami, A., 1973. Glial fibrillary acidic protein from normal human brain. Purification and properties. Brain Res. 57, 343–360.

Dahl, D., Crosby, C.J., Gardner, E.E., Bignami, A., 1982. Purification of the glial fibrillary acidic protein by anion-exchange chromatography. Anal. Biochem. 126, 165–169.

Dubovik, V., Faigon, M., Feldon, J., Michaelson, D.M., 1993. Decreased density of forebrain cholinergic neurons in experimental autoimmune dementia. Neuroscience 56, 75–82.

Finney, M., 1995. Molecular cloning of PCR products. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), Current Protocols in Molecular Biology. Wiley, New York, pp. 15.17.11–15.17.13.

Heinonen, O., Syrjänen, S., Soininen, H., Talasniemi, S., Kaski, M., Mäntyjärvi, R., Syrjänen, K., Riekkinen Sr., P., 1993. Circulating immune complexes in sera from patients with Alzheimer's disease, multi-infarct dementia and Down's syndrome. Neurosci. Lett. 149, 67–70.

Hunkapiller, M.W., Lujan, E., Ostrander, F., Hood, L.E., 1984. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. 91, 227–236.

Inagaki, M., Nakamura, Y., Takeda, M., Nishimura, T., Inagaki, N., 1994. Glial fibrillary acidic protein: dynamic property and regulation by phosphorylation. Brain Pathol. 4, 239–243.

Janzer, R.C., Raff, M.C., 1987. Astrocytes induce blood–brain barrier properties in endothelial cells. Nature 325, 253–257.

Kaneko, K., Seki, K., Ishida, K., Uchihara, T., Kanda, T., Inuzuka, T., Miyatake, T., 1993. Circulating autoantibody to mature neurons and astrocytes of humans and some mammals present in a demented patient with autoimmune disorder. Eur. Neurol. 33, 450–453.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Liedtke, W., Edelmann, W., Bieri, P.L., Chiu, F.-C., Cowan, N.J., Kucherlapati, R., Raine, C.S., 1996. GFAP is necessary for the integrity of CNS white matter architecture and long-term maintenance of myelination. Neuron 17, 607–615.

Liem, R.K.H., Shelanski, M.L., 1978. Identity of the major protein in "native" glial fibrillary acidic protein preparation with tubulin. Brain Res. 145, 196–201.

Manthorpe, M., Rudge, J.S., Varon, S., 1986. Astroglial cell contributions to neuronal survival and neuritic growth. In: Fedoroff, S., Vernadakis, A. (Eds.), Astrocytes, vol. 2. Academic Press, New York, pp. 315–376.

Mecocci, P., Parnetti, L., Donato, R., Santucci, C., Santucci, A., Cadini, D., Foa, E., Cecchetti, R., Senin, U., 1992. Serum autoantibodies against glial fibrillary acidic protein in brain aging and senile dementia. Brain Behav. Immun. 6, 286–292.

Mecocci, P., Parnetti, L., Romano, G., Scarelli, A., Chionne, F., Cecchetti, R., Polidori, M.C., Palumbo, B., Cherubini, A., Senin, U., 1995. Serum anti-GFAP and anti-S100 autoantibodies in brain aging, Alzheimer's disease and vascular dementia. J. Neuroimmunol. 57, 165–170.

Pekny, M., Levéen, P., Pekna, M., Eliasson, C., Berthold, C.-H., Westermark, B., Betsholtz, C., 1995. Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. EMBO J. 14, 1590–1598.

Posner, J.B., 1991. Paraneoplastic syndromes. Neurol. Clin. 9, 919–936.

Reeves, S.A., Helman, L.J., Allison, A., Israel, M.A., 1989. Molecular cloning and primary structure of human glial fibrillary acidic protein. Proc. Natl. Acad. Sci. USA 86, 5178–5182.

Rich, J.B., Rasmusson, D.X., Folstein, M.F., Carson, K.A., Kawas, C., Brandt, J., 1995. Nonsteroidal anti-inflammatory drugs in Alzheimer's disease. Neurology 45, 51–55.

Rogers, S.W., Andrews, P.I., Gahring, L.C., Whisenand, T., Cauley, K., Crain, B., Hughes, T.E., Heinemann, S.F., McNamara, J.O., 1994. Autoantibodies to glutamate receptor GluR3 in Rasmussen's encephalitis. Science 265, 648–651.

Rueger, D.C., Dahl, D., Bignami, A., 1978. Comparison of bovine glial fibrillary acidic protein with bovine brain tubulin. Brain Res. 153, 188–193.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A Laboratory Manual, second ed. Cold Spring Harbor Press, New York.

Serot, J.M., Béné, M.C., Gobert, B., Christmann, D., Leheup, B., Faure, G.C., 1992. Antibodies to choroid plexus in senile dementia of Alzheimer's type. J. Clin. Pathol. 45, 781–783.

Shibuki, K., Gomi, H., Chen, L., Bao, S., Kim, J.J., Wakatsuki, H., Fujisaki, T., Fujimoto, K., Katoh, A., Ikeda, T., Chen, C., Thompson, R.F., Itohara, S., 1996. Deficient cerebellar long-term depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. Neuron 16, 587–599.

Smith, D.B., Corcoran, L.M., 1994. Expression and purification of glutathione-S-transferase fusion proteins. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), Current Protocols in Molecular Biology. Wiley, New York, pp. 16.17.11–16.17.17.

Steinert, P.M., Roop, D.R., 1988. Molecular and cellular biology of intermediate filaments. Annu. Rev. Biochem. 57, 593–625.

Tanaka, J., Murakoshi, K., Takeda, M., Kato, Y., Tada, K., Hariguchi, S., Nishimura, T., 1988. A high level of anti-GFAP autoantibody in the serum of patients with Alzheimer's disease. Biomed. Res. 9, 209–216.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

Weinstein, D.E., Shelanski, M.L., Liem, R.K.H., 1991. Suppression by antisense mRNA demonstrates a requirement for the glial fibrillary acidic protein in the formation of stable astrocytic processes in response to neurons. J. Cell Biol. 112, 1205–1213.

Yamada, M., Zurbriggen, A., Oldstone, M.B.A., Fujinami, R.S., 1991. Common immunologic determinant between human immunodeficiency virus type 1 gp41 and astrocytes. J. Virol. 65, 1370–1376.